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Abstract: An enzyme-based cyclic pathway for trans to cis isomerization of the chromophore of visual pigments (11-cis-retinal) is intrinsic to vertebrate cone and rod vision. This process, called the visual cycle, is mostly characterized in rod-dominated retinas and essentially depends on RPE65, an all-trans to 11-cis-retinoid isomerase. Here we analysed the role of RPE65 in zebrafish, a species with a cone-dominated retina. We cloned zebrafish RPE65 and showed that its expression coincided with photoreceptor development. Targeted gene knockdown of RPE65 resulted in morphologically altered rod outer segments and overall reduced 11-cis-retinal levels. Cone vision of RPE65-deficient larvae remained functional as demonstrated by behavioural tests and by metabolite profiling for retinoids. Furthermore, all-trans retinylamine, a potent inhibitor of the rod visual cycle, reduced 11-cis-retinal levels of control larvae to a similar extent but showed no additive effects in RPE65-deficient larvae. Thus, our study of zebrafish provides in vivo evidence for the existence of an RPE65-independent pathway for the regeneration of 11-cis-retinal for cone vision.

DOI: https://doi.org/10.1111/j.1460-9568.2007.05801.x

Evidence for RPE65-independent vision in the cone-dominant zebrafish retina

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Running Title: Cone Visual Pigment Regeneration

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ABSTRACT

An enzyme-based cyclic pathway for *trans* to *cis* isomerization of the chromophore of visual pigments (11-*cis*-retinal) is intrinsic to vertebrate cone and rod vision. This process, called the visual cycle, is mostly characterized in rod-dominated retinas and essentially depends on RPE65, an all-*trans* to 11-*cis*-retinoid isomerase. Here we analyzed the role of RPE65 in zebrafish, a species with a cone-dominated retina. We cloned zebrafish *RPE65* and showed that its expression coincided with photoreceptor development. Targeted gene knock-down of RPE65 resulted in morphologically altered rod outer segments and overall reduced 11-*cis*-retinal levels. Cone vision of RPE65-deficient larvae remained functional as evidenced by behavioral tests and by metabolite profiling for retinoids. Furthermore, all-*trans* retinylamine, a potent inhibitor for the rod visual cycle, reduced 11-*cis*-retinal levels of control larvae to a similar extend, but showed no additive effects in RPE65-deficient larvae. Thus, our study of zebrafish provides *in vivo* evidence for the existence of an RPE65-independent pathway for the regeneration of 11-*cis*-retinal for cone vision.

INTRODUCTION

The vertebrate visual system employs two types of photoreceptors working at different light intensities. Rods are specialized for vision under dim light conditions, whereas cones are active under daylight conditions, enabling high-resolution color vision (Rodieck, 1998). Both rod and cone visual pigments are of bipartite structure, consisting of a transmembrane protein (opsin) and the vitamin A-derived chromophore 11-*cis*-retinal (RAL)[1] (Palczewski, 2006). Light absorption leads to isomerization of the chromophore from 11-*cis* to all-*trans* configuration resulting in the metarhodopsin state, which activates a signal transduction cascade (Burns & Baylor, 2001). Finally, the visual pigment decays into the opsin moiety and all-*trans* RAL, which must be recycled to 11-*cis*-RAL to support continuous vision (McBee *et al.*, 2001; Lamb & Pugh, 2004).

Detailed knowledge about this process, called the visual (retinoid) cycle (Wald, 1968), stems from work on species with rod-dominated retinas such as mice. Here, the visual cycle (for rods) involves two cellular compartments, the rod outer segments (ROS) and the closely associated retinal pigment epithelium (RPE). The re-isomerization to the 11-*cis* conformation of the visual chromophore is achieved by a two-step enzymatic reaction in the RPE (Bernstein *et al.*, 1987; Rando, 1991). Thereby, the action of lecithin retinol acyltransferase on all-*trans* ROL generates retinyl esters (RE), which are further processed into 11-*cis*-retinol (ROL) by an isomerase. The latter enzyme was recently identified as retinal pigment epithelium-specific 65 kDa protein (RPE65), an abundant protein of the RPE (Jin *et al.*, 2005; Moiseyev *et al.*, 2005; Redmond *et al.*, 2005).

Cones are operative under bright light, under which rods are saturated but consume 11-*cis*-RAL. This condition might necessitate a cone-specific regeneration pathway to avoid competition for 11-*cis*-RAL between the two types of photoreceptors. In mice, a *RPE65* null mutation results in 11-*cis*-retinal deficiency and results in severely impaired cone and rod function (Seeliger *et al.*, 2001). In contrast, in

cone-dominated animals such as chicken evidence has been provided for an additional cone specific pathway for the recycling of the visual chromophore. Recent biochemical studies showed that this pathway probably takes place in cone photoreceptors and Müller glia cells (Mata *et al.*, 2002) and thus is likely RPE65-independent.

In vivo investigations on the role of RPE65 in cone-dominated animals have been hampered by a lack of a suitable model organism. In this study, we used the larva of the zebrafish (*Danio rerio*), which is excellently suited for genetic and pharmacological manipulations. Even though cone and rod photoreceptors develop concomitantly (Schmitt & Dowling, 1996), the retina is functionally cone-dominated at early larval stages (Bilotta *et al.*, 2001). We cloned gene orthologues for *RPE65* and showed that one of these is expressed in the RPE of the larval eyes. We then disrupted the RPE65-dependent visual cycle by genetic and pharmacological manipulation by morpholino oligonucleotides (MO) (Nasevicius & Ekker, 2000) and Ret-NH_2 (Golczak *et al.*, 2005b). Targeted gene knock-down of RPE65 resulted in morphologically altered rod outer segments (ROSs) and overall reduced 11-*cis*-RAL levels. Ret-NH_2 reduced 11-*cis*-RAL levels to a similar extent, but showed no additive effects in RPE65-deficient larvae. Under all conditions, the residual 11-*cis*-RAL was efficiently regenerated and cones remained functional as evidenced by behavioral tests.

MATERIAL AND METHODS

Fish maintenance and strains - Zebrafish were bred and maintained under standard conditions at 28°C (Westerfield, 1994). Embryos from the AB/TL strain were used for the expression analyses. Morphological features characteristic of developmental stages were used to determine the stage of the embryos in hours post-fertilization (hpf), according to (Kimmel *et al.*, 1995). Embryos used for *in situ* hybridization experiments were raised in the presence of 200 μM 1-phenyl-2-thiourea (Sigma) to inhibit pigmentation.

Cloning of RPE65a and RPE65b - Total RNA was isolated from adult zebrafish eyes by using the RNAeasy Kit (QIAGEN, Germany) according to the manufacturer's instructions. cDNA was synthesized by using the SuperScript II Reverse Transcriptase Kit (Invitrogen, Germany) as described in the user's manual. The *RPE65a* cDNA was cloned by RT-PCR using degenerated primers designed against a 519-bp fragment of cDNA deduced from highly conserved amino acid sequences: TGGGGAGCNAAYTAYATGGA, GGYTCYTGCCANAYCCA. The PCR product was cloned into SK-pBluescript (Invitrogen, Germany) and sequenced with T7 and T3 primers. Additionally, a zebrafish cDNA clone (GenBank Accession No. AI942672, AI884144) encoding a putative *RPE65* gene was identified by searching the EST database. The clone was obtained from the RZPD (Resource Center/Primary Database, Germany) regrown and purified. Finally, the clone was sequenced with M13 reverse primer, M13(-20) forward primer, and the full length of the clone was obtained by sequencing using gene-specific primers: AGGTTTTTCACTTACTTTCAAGG, CCACATTAACTGTTTTGAAGACC, ACTTCCTGGAGCATCAGAGG, AGGACGTCAACCTCCGCACG. The sequencing of the EST resulted in a second *RPE65* cDNA, named *RPE65b*. To obtain the 5' and 3' cDNA-sequences of the two genes, RACE-PCRs were carried out with the SMART RACE cDNA Amplification Kit (Clontech, Germany). The RACE primers used

to amplify the 5'primer region of *RPE65a* were GCTGAAGTTTCTGAGCGCCTGGAGCA and for the nested PCR GCCATCCTACGTGCACAGTTTTGGGA. The primers used to amplify the 3'primer region of *RPE65a* were TGCTCCAGGCGCTCAGAAACTTCAGC and TCCCAAAACTGTGCACGTAGGATGGC. To obtain the 5'primer region of *RPE65b* also nested PCR was carried out using the following primers: AACCGACAGATAATTGCAGAGGTCCACC and for the nested PCR GTTTCCAGGGTATCAGGATTGACTTTGG. The fragments obtained were then cloned into the pCR2.1-vector (Clontech, Germany) and pCRII-TOPO vector (Invitrogen, Germany), respectively. The constructs were sequenced using M13 reverse primer, M13 (-20) forward primer. Accession Numbers (GenBank) were as follows: zebrafish RPE65a, AY646886; zebrafish RPE65b, AY646887.

Whole mount in situ hybridization and immunostaining **-** Whole-mount *in situ* hybridization was performed as previously described (Hauptmann & Gerster, 1994), using *RPE65a*, *RPE65b* and *rhodopsin* as probes. *Rhodopsin* antisense RNA probe used for *in situ* hybridization was a gift from Dr. Wolfgang Driever (University of Freiburg, Germany). Whole mount antibody labeling was performed according to Solnica-Krezel and Driever (Solnica-Krezel & Driever, 1994), except that the methanol fixation step was omitted. For RPE65 staining, an antiserum raised against recombinant zebrafish RPE65a was used in a dilution of 1:300. For this purpose, recombinant zebrafish RPE65a was purified upon heterologous expression in *E. coli*. One milligram purified RPE65a protein was used to raise a polyclonal antiserum in rabbits (Eurogentec, Brussels, Belgium). For secondary-antibody staining, Cy3 goat anti-rabbit IgG (Sigma, Germany; 1/500) was used.

Immunohistochemistry - Fixed larvae were cryoprotected in 30% sucrose overnight. Entire larvae were embedded in cryomatrix (JUNG; Tissue Freezing Medium), and rapidly frozen in liquid N2; 20-µm-thick sections were cut at -20 °C, mounted on superfrost slides and air dried at 37 °C for 2 h. The slides were stored at -20 °C until further use. For immunohistochemistry, slides were thawed, washed three

times in phosphate-buffered saline (PBS; 50 mM), pH 7.4, and incubated in 20% NGS, 2% BSA in 0.3% PBS / Triton X-100 (PBST) for 1 h. For double cone and rod labeling, sections were incubated with anti-mouse zpr1 (Zebrafish International Resource Center, Eugene, USA) 1:20 and 1D1 (kindly provided by Drs Ann Morris and Jim Fadool, Florida State University, Tallahassee, USA) 1:100, respectively. The immunoreaction was visualized by using Alexa Fluor 488 anti-mouse IgG (Molecular Probes, Leiden, Netherlands) 1:1000 as a secondary antibody. For all immunocytochemical experiments, negative controls were carried out in the same way but without using the first antibody. Slides were viewed with an Olympus BX61 compound microscope (Olympus, Hamburg, Germany). The obtained images were processed using Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA).

Immunoblotting **–** Immunoblot analysis was performed with zebrafish protein extracts using polyclonal serum raised against murine RPE65 (Wenzel *et al.*, 2005) at a 1:300 dilution. As loading control antiserum raised against beta-tubulin (Chemicon;1/1000) was used. Immunoblots were developed with the ECL-system (Pharmacia, Germany). Quantification was performed by the Quantity-one (version 4.6) software (Biorad, Germany).

Morpholino injections **-** *RPE65* antisense morpholino 5´-CAGGGTGTTCAAAACGGCTGACCAT-3´ (GeneTools, LLC) in 0.3x Danieau´s solution (1x Danieau: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes, pH 7.6) to obtain a stock concentration of 1 mM. Embryos were injected at the one-cell stage at about 8 ng per zygote. Control morpholinos from Gene tools were injected with the same concentration.

Light treatments of zebrafish larvae - For all illumination experiments, we used a KL 1500-T electronic fiber optic device (Schott, Germany). Before illumination (10,000 lux, bright white light), zebrafish larvae were placed into Petri dishes under dim red light. The bottom and the sides of these dishes were coated with light reflecting aluminum foil. Upon the different illumination regimens, larvae were immediately collected, killed on ice, and stored at -70°C prior to HPLC-analysis.

Expressing of RPE65 and LRAT in Sf9 cells and isomerization assay – Human RPE65 and mouse LRAT cDNA was cloned into pFastBac HT B vector (Invitrogen) using EheI, HindIII and BamH1, HindIII restriction sites, respectively. Bac-to-Bac baculovirus expression system (Invitrogen) was employed to generate recombinant bacmids and produce high-titer baculoviruses stocks. Sf9 cells were cotransfected with P2 stock of baculoviruses encoding RPE65 and LRAT. Two days postinfection expression of recombinant proteins was verified by immunoblotting using anti-human REP65 antibodies (Novus) and anti-mouse LRAT antibodies (Moise *et al*., 2007). For the *in vivo* isomerization assays, cells were grown to confluence in 6 well plates and infected 48 h prior to experiment. The medium was replaced for fresh one containing 5% FBS and 10 μM of all-*trans*-retinol. In case of inhibition the medium was supplemented with all-*trans*-retinyl amine in final concentration of 3 μM. Cells were incubated in dark for 18h, harvested with the medium, mixed with 2 ml of methanol and extracted with 4 ml of hexane. Organic fraction was dried down in speed-vac, redissolved in 250 μl of hexane and analyzed on normal phase HPLC (Hewlett Packard 1100) equipped with a diode array detector and Agilent-Si column (4.6 x 250 mm, 5 μm). Retinoids were separated in 10% ethylacetate in hexane at flow rate 1.4 ml/min.

Ret-NH_2 synthesis and treatments - all-*tran*s-Ret-NH_2 was synthesized by reacting all-*trans*-RAL with ammonia under anhydrous conditions followed by reduction with excess of sodium borohydride as described elsewhere (Golczak *et al.*, 2005b). Ret-NH_2 was purified by flash liquid chromatography on silica gel and the purity of Ret-NH_2 was confirmed by normal and reverse phase HPLC analysis. Prior to the experiments Ret-NH_2 was dissolved in ethanol and added to the egg-water of 5 dpf larvae to obtain final concentrations as indicated in the Result section. Upon 30 min of incubation under dim red light, larvae were subjected to illumination by bright light (10,000 lux for 20 min) and dark-readapted for 45 min prior to HPLC analysis.

Behavioral assays - Visual behavior was assayed as described in (Rinner *et al.*, 2005b).

HPLC separation for retinoids - For retinoid analysis, larvae were immediately transferred to ice water after light treatment. Retinoids were extracted under monochromatic light (695 nm) either from the anterior heads and subjected to HPLC separation as previously described (Lampert *et al.*, 2003). For quantification of the molar amounts of retinoids, peak integrals were scaled with defined amounts of reference substances from Sigma-Aldrich (Germany) and were quantified using the 32 Karat software (Beckman Instruments). Student's *t*-test was used for statistical analysis.

RESULTS

RPE65 orthologous genes are expressed in the developing eyes and the pineal gland - With its genetic accessibility and its early maturing visual system the zebrafish larva presents a unique model to analyze cone vision. To exploit this, we cloned two *RPE65* genes, which we denoted as *RPE65a* and *RPE65b*. The deduced proteins are highly homologous to human RPE65 showing 74% and 75% amino acid sequence identity, respectively. As revealed by RNA *in situ* hybridization, only *RPE65a* was expressed in light sensitive structures during zebrafish development (Figure 1A-C). The *RPE65a* transcript was expressed already at 16 hours post fertilization (hpf) in the pineal gland, a light-sensitive endocrine organ (Figure 1A). Expression in the RPE was initiated in the ventro-nasal patch at 40 hpf, preceding photoreceptor differentiation by a few hours (Figure 1B). During subsequent development, expression in the RPE became stronger and more widespread, covering the entire RPE except the cilliary margin by 56 hpf (Figure 1C). In cross sections it became clear that retinal *RPE65a* expression was confined to RPE cells (Figure 1D). By contrast, *RPE65b* mRNA was detectable in migrating neural crest cells during somitogenesis (Fig. 1E). At early larval stages, *RPE65b* mRNA expression was visible in the ventricular zone, the upper and lower jaw and in the developing pectoral fins. At later larval stages, *RPE65b* mRNA expression faded out (Fig. 1H).

Generation of RPE65-deficient zebrafish larvae - In order to address the role of RPE65 for cone vision, we performed a loss-of-function study using a morpholino (MO)-based approach (Nasevicius & Ekker, 2000). This technique is routinely used for embryonic stages, but has also been successfully applied to achieve gene knockdown at early larval stages (Rinner *et al.*, 2005a; Seiler *et al.*, 2005). We designed a MO which specifically blocked the initiation of translation of *RPE65a* in a coupled *in vitro* transcription / translation system (data not shown). We then injected this MO into eggs of the 1-cell

stage embryo and determined RPE65 protein levels in the heads of 4 and 5 dpf larvae. We used an antiserum, which detected both recombinant RPE65a and RPE65b, to determine RPE65 protein levels by immunoblot analysis. We quantified RPE65 protein levels by densitometry and normalized it by the beta-tubulin loading control. In morphants, we found a reduction of minimally 97 % (4 dpf) and 88 % (5 dpf) of the RPE65 protein levels of controls (Figure 2A,B). Similar results were obtained in three independent experiments. Additionally, we confirmed RPE65-deficiency in the eyes of 5dpf morphant larvae by whole mount immunohistochemistry (n = 20; Figure 2C,D).

Analyses of the eyes of RPE65-deficient larvae - To address the consequences of RPE65-deficiency for the larval eyes, we examined standard histological sections. We found a normal lamination of the distinct retinal layers (Figure 3A,B). *In situ* hybridization for *rhodopsin* mRNA showed that its expression pattern was comparable in both morphants and controls with the most abundant staining of the dorsal and ventral parts of the retina (Figure 3C,D). We then performed immunostainings against double cones (zpr1-staining) and rods (1D1-staining) to determine whether the distribution and/or morphology of the photoreceptors was affected in RPE65-deficient animals (n = 5: Figure 4). Double cone distribution and morphology was identical in the control and MO-treated larvae (Figure 4 A,C). However, even though the distribution of the rods throughout the retina remained unaffected in the morphants, with numerous labeled cells in the ventral and dorsal retina and only few labeled cells in the central part of the retina, the morphology of the photoreceptors was altered (Figure 4B,D). In a close-up of the dorsal retina, the photoreceptors of control larvae showed the characteristic morphology with rod spherules as well as healthy outer segments (Figure 4B'). In contrast, RPE65-deficient larvae showed deteriorating rod outer segments that break up into several small particles, presumptive phagosomes (Figure 4D'). Thus, rod photoreceptor function is likely impaired in RPE65a-deficient larvae.

Regeneration of 11-cis-RAL takes place in RPE65-deficient larvae - In order to assess the effect of RPE65-deficiency on ocular retinoid metabolism, we performed HPLC-analyses. To approach this issue, morphants and control siblings were raised under a standard day-and-night light regime (14/10). These larvae were then dark-adapted over night, divided into three groups (n = 50 per group) and subjected to different illumination regimes. In the first group, retinoid analysis was immediately performed. The second group was bleached by exposure to bright light (10,000 lux) for 20 min. The third group was first bleached and then dark-readapted for 45 min prior to HPLC-analysis.

The levels of 11-*cis*-RAL were significantly reduced already in dark-adapted RPE65-deficient larvae consistent with morphological alterations in ROS. In both morphants and controls, the amounts of 11-*cis*-RAL decreased upon bleaching with bright light. Surprisingly, dark-readaptation for 45 min resulted in regeneration of 11-*cis*-RAL to the initial amounts found in the non-bleached groups. Thus, despite RPE65-deficiency 11-*cis*-RAL existed and was regenerated after bleaching with bright light in repeated experiments (n = 3).

In the eyes of both morphants and controls, we found high levels of RE (Table 1). RE accumulation has been reported in *RPE65*$^{-/-}$ mice (Redmond *et al.*, 1998). Since high levels of RE also existed in controls, but also in the eyes of adult zebrafish (Schonthaler *et al.*, 2005), we conclude that this characteristic is not related to RPE65-deficiency.

Besides these retinoids, we detected in both morphants and controls all-*trans* RAL and trace amounts of all-*trans* ROL. However, 9-*cis*-RAL was not detectable in RPE65-deficient larvae, which exists in *RPE65*$^{-/-}$ mice and recombines with opsin to form isorhodopsin in the absence of 11-*cis*-RAL (Fan *et al.*, 2003).

Ret-NH_2 has no effects on 11-cis-RAL regeneration in RPE65-deficient larvae - To exclude that residual level of RPE65 in morphant larvae might be responsible for 11-*cis*-RAL regeneration, we performed repeated bleaching experiments and again determined 11-*cis*-RAL levels as a measure for the visual pigment content. For this purpose we subjected 5 dpf morphant and control larvae four-times to a 20 min bright light illumination (10,000 lux for 20 min), each time followed by a 10 min dark interval. In repeated experiments (n = 3), HPLC-analysis revealed that visual pigments were not bleached out since 11-*cis*-RAL still was detectable in both morphant and control larvae in comparable amounts as found upon a single 20 min light bleach (see Table 1 and data not shown).

We then took advantage of Ret-NH_2. This compound has been recently shown to act as a potent pharmacological inhibitor of the rod retinoid cycle (Golczak *et al.*, 2005b). To demonstrate that Ret-NH_2 directly inhibits Rpe65 enzymatic activity, we established a cell culture system. For this purpose, we co-transfecetd insect *SF9* cells with expression vectors for Lrat and Rpe65 and verified protein expression by immunoblot analysis (Fig. ??A). To measure Rpe65 enzymatic activity in the presence and absence of Ret-NH_2, we incubated cells with all-*trans* retinol. Upon incubation with all-*trans* retinol alone, these cells produced significant amounts of 11-*cis* retinol (Fig. ??B,C).. In contrast, cells incubated with both all-*trans* retinol and Ret-NH_2 failed to produce 11-*cis* retinol (Fig. ?? B,C). Thus, Ret-NH_2 can inhibit Rpe65 enzymatic activity.

We then took advantage of Ret-NH_2, a potent pharmacological inhibitor of the RPE65-dependent retinoid cycle (Golczak *et al.*, 2005b). We first tested the effects of Ret-NH_2 treatment in wild-type larvae. For this purpose, we added Ret-NH_2 (5 µM final concentration) into the water and incubated the larvae for 30 min under dim red light. We then exposed the larvae to bright light illumination (10,000 lux for 20 min) and determined the 11-*cis*-RAL levels upon 45 min dark-readaption. Under this condition, 11-*cis*-RAL levels were significantly reduced as compared to none-treated larvae, but visual pigments were not bleached out (Figure 4A). No further reduction of 11-*cis*-RAL levels was observed

when we doubled the concentration of the inhibitor (data not shown). While 11-*cis*-RAL levels decreased in Ret-NH_2 –treated larvae, ROL levels and RE levels increased, most probably due to a deamination of Ret-NH2 to ROL and subsequent esterification (Golczak *et al.*, 2005a). Additionally, N-retinylamides became detectable (Figure 4B), which are formed by the action of Lrat from Ret-NH_2 (Golczak *et al.*, 2005a). Interestingly, Ret-NH_2 treatment caused a comparable reduction of 11-*cis*-RAL, approximately 50 % levels in control larvae, as we observed upon the targeted gene knock down of RPE65 (Figure 4B). We then asked whether a combination of both Ret-NH_2- and MO-treatments impairs 11-*cis*-RAL regeneration upon bright light illumination. To employ this, we subjected RPE65-deficient larvae to Ret-NH_2 treatments, but we found no further reduction of 11-*cis*-RAL levels upon illumination (10,000 lux for 20 min) and determination of the 11-*cis*-RAL levels upon 45 min dark-readaption (Figure 4A). Thus, both MO and/or Ret-NH_2 treatments led to similar reduction in 11-*cis*-RAL levels in the larval eyes. Since no additive effects were observed, we concluded that already the MO treatment efficiently blocked the RPE65-dependent visual cycle. More interestingly, the residual 11-*cis*-RAL was not bleached out under any condition in repeated experiments (n = 3). This observation indicates that RPE65-independent pathways must exist for the regeneration of the visual chromophore in the zebrafish eyes.

Behavioral consequences of RPE65-deficiency **-** To directly assess the consequences of RPE65-deficiency on visual performance, we employed a vision-dependent behavioral test. This test is based on the optokinetic response that is triggered by movement in the visual field. The ratio of eye movement velocity to the stimulus pattern velocity (gain) is related to stimulus properties such as contrast, brightness, spatial frequency, and temporal frequency (22). To compare visual performance between RPE65-deficient larvae and control larvae a contrast sensitivity function was measured where spatial frequency (a measure of stripe width) of a moving grating stimulus was varied. This assay examines visual resolution and contrast sensitivity. Visual performance was not affected in RPE65-

deficient morphants as compared to controls under moderate mean luminous intensity (120 cd/m^2) (Figure 7A). In the next experiment we challenged retinoid recycling by measuring contrast sensitivity under dark conditions (0.36 cd/m^2) and with pre-adaptation to bright light (386 cd/m^2). Contrast sensitivity is indistinguishable for RPE65-deficient and control larvae when measured with a dark grating, while it is slightly reduced under bright light conditions (Figure 7B).

DISCUSSION

RPE65 has been recently identified to encode the long-searched all-*trans* to 11-*cis*-retinoid isomerase in vertebrates (Jin *et al.*, 2005; Moiseyev *et al.*, 2005; Redmond *et al.*, 2005). Here we analyzed the role of RPE65 in zebrafish, a species with a cone-dominated retina. For this purpose, we cloned two *RPE65* orthologous genes. We found that only *RPE65a* was expressed during development spatiotemporally coinciding with the course of photoreceptor development. We disrupted RPE65a by a MO-mediated targeted knock-down to analyze its function in ocular retinoid metabolism. RPE65-deficient larvae possessed significantly reduced levels of 11-*cis*-RAL. Examination of the outer retina in RPE65-deficient larvae by immunohistochemistry revealed unaffected cone morphology and distribution but shortened and deteriorated rod outer segments, while the number and distribution of rods remained unaffected. Thus, RPE65-deficiency interferes with rod photoreceptor function in zebrafish as has been previously described for *RPE65*$^{-/-}$ mice (Redmond *et al.*, 1998).

In contrast to the mouse model (Seeliger *et al.*, 2001), RPE65-deficiency did not interfere with cone function. We found sufficient 11-*cis*-RAL to sustain visual performance under bright light conditions. The recent biochemical characterization of RPE65 revealed that its catalytic activity is quite low (Jin *et al.*, 2005; Moiseyev *et al.*, 2005; Redmond *et al.*, 2005). This may explain the large abundance of RPE65, constituting about 10% of total microsomal proteins in bovine RPE (Bavik *et al.*, 1992) and the long time course (45 min in mice) for rod visual pigment regeneration upon bleaching (Wenzel *et al.*, 2001). Due to these biochemical characteristics of RPE65, we exclude that its low residual amounts in the morphants mediate cone function. We provide further evidence for this assumption by the use of Ret-NH_2 a potent pharmacological inhibitor of the visual cycle (Golczak *et al.*, 2005b). In cell culture, we first demonstrated that Ret-NH_2 can inhibit Rpe65 enzymatic activity. Interestingly, Ret-NH_2 treatments reduced 11-*cis*-RAL levels in control larvae to a similar extend as

gene knock down of RPE65. Most strikingly, Ret-NH_2 had no additive effects in RPE65-deficient larvae indicating that already the MO-treatment efficiently blocked the RPE65-dependent visual cycle.

Thus, we provide evidence that an additional pathway for 11-*cis*-RAL generation exists in the zebrafish eyes. Our data best fit a model in which visual pigments can be recycled by two independent pathways. One likely represents the established RPE65-dependent visual cycle. In parallel, another retinoid cycle supports cone vision. The existence of this additional pathway is supported by previous data implicating Müller cells in the recycling of the visual chromophore (Goldstein & Wolf, 1973; Hood & Hock, 1973; Saari *et al.*, 1982; Bunt-Milam & Saari, 1983; Saari & Bredberg, 1987). Mata et al. (Mata *et al.*, 2002) have recently provided a model for such a cone specific pathway that accommodates the elevated demand for 11-*cis*-RAL regeneration under photopic conditions. In contrast to the established visual cycle of rods, this visual cycle likely involves Müller glia cells and depends on a novel type of all-*trans* to 11-*cis*-retinol isomerase (Mata *et al.*, 2005). The enzymatic activities of the proposed Müller cell-based pathway could not be detected in retina protein extracts from mice (Mata *et al.*, 2002). This difference readily explains that RPE65 gene knock out also affects cone function in this rod-dominated animal (Seeliger *et al.*, 2001). However, there is evidence for all-*trans* to 11-*cis* retinoid isomerases other than RPE65 also in mice. Tu et al. (Tu *et al.*, 2006) have recently reported that melanopsin-dependent photoreception in the inner retina is independent of the RPE65-dependent visual cycle and not susceptible to Ret-NH_2 treatments. Melanopsin resembles in several respects visual pigments of insects, which lack RPE65 (von Lintig *et al.*, 2001). Here, both light-dependent and light-independent mechanisms for the production of 11-*cis*-RAL or 11-*cis*-RAL derivatives have been described (Ozaki *et al.*, 1993).

The need for the evolution of cone and rod specific regeneration mechanisms in animals with high resolution color vision may have arisen due to a competition for 11-*cis*-RAL between these two types of photoreceptors, which work at different light intensities. This implies that the existence of two

visual cycles, an RPE65-based and a possibly Müller cell-based one, might not be limited to cone-dominant species. Humans, for example, have an excess of rods but use cones, particularly in the fovea, for high-resolution color vision. The observation that children suffering from Leber congenital amaurosis caused by mutations in *RPE65* are able to see color before losing sight (Lorenz *et al.*, 2000; Paunescu *et al.*, 2004) supports the existence of an RPE65-independent regeneration pathway for cone visual pigments. The blindness of these patients might be a secondary consequence caused by the degeneration of rod photoreceptors due to 11-*cis*-RAL deficiency (Woodruff *et al.*, 2003) or could result from fragility of cones when unliganded (Rohrer *et al.*, 2005; Znoiko *et al.*, 2005).

In summary, our study supports the idea of the existence of a cone-specific visual cycle. As represented here, the genetic accessibility in conjunction with appropriate techniques establishes the zebrafish as a valuable model to elucidate this pathway in detail.

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FOOTNOTES

The authors would like to thank Beate Ziser (University of Freiburg) for skillful technical assistance with HPLC analysis. This work was supported by the German Research Council (JvL; DFG LI956-2), the Ministry of Science, Research and the Arts, Baden-Württemberg (JvL), the Swiss National Science Foundation (HBS, MG, SCFN), Velux Foundation (SCFN), the EMBO Young Investigator Program (SCFN), a ZNZ student fellowship (OR), the Roche Research Foundation (OR), and ETH internal grants (HBS). SCFN was a SNF Förderungsprofessor (PP00A-68868). This research was also supported, in part, by NIH grant EY09339 and P30 EY11373. We thank Dr. Martin Schwab for helpful comments and financial support. Murine antisera against RPE65 were kindly provided by Dr. Andreas Wenzel.

[1]The abbreviations used are: RAL: retinal; RPE: retinal pigment epithelium; ROL: retinol; RE: retinyl ester; MO: morpholino oligonucleotide; Ret-NH_2: all-*trans* retinyl amine, ROS: rod outer segments; hpf: hours post fertilization; dpf: days post fertilization; bp: base pairs, EST: expressed sequencing tag; HPLC: high performance liquid chromatography.

TABLE 1

Retinoid composition of the eyes of 5 dpf larvae subjected to different light conditions. Retinoids were extracted from 50 larval heads under dim red light and subjected to HPLC-analysis. The value (pmol / eye pair) give the means of three independent experiments ± SD. Dark, dark-adapted over night; bleached, dark-adapted over night and illuminated with 10,000 lux for 20 min; bleached/dark, dark-adapted over night and illuminated with 10,000 lux for 20 min and dark-readapted for 45 min. control, WT larvae; MO, RPE65a knock down larvae.

		RE	**11-*cis*-RAL**	**all-*trans* RAL**	**all-*trans* ROL**
dark	control	4.63 ± 0.71	2.82 ± 0.18	0.46 ± 0.03	0.04 ± 0.01
	MO	4.5 ± 0.72	1.52 ± 0.08	0.18 ± 0.01	0.08 ± 0.01
bleached	control	6.6 ± 1.19	1.65 ± 0.02	0.34 ± 0.01	0.11 ± 0.01
	MO	6.09 ± 1.08	0.53 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
bleached /dark	control	6.28 ± 1.42	2.81 ± 0.18	0.36 ± 0.02	0.01 ± 0.01
	MO	5.27 ± 1.14	1.40 ± 0.07	0.40 ± 0.02	0.11 ± 0.01

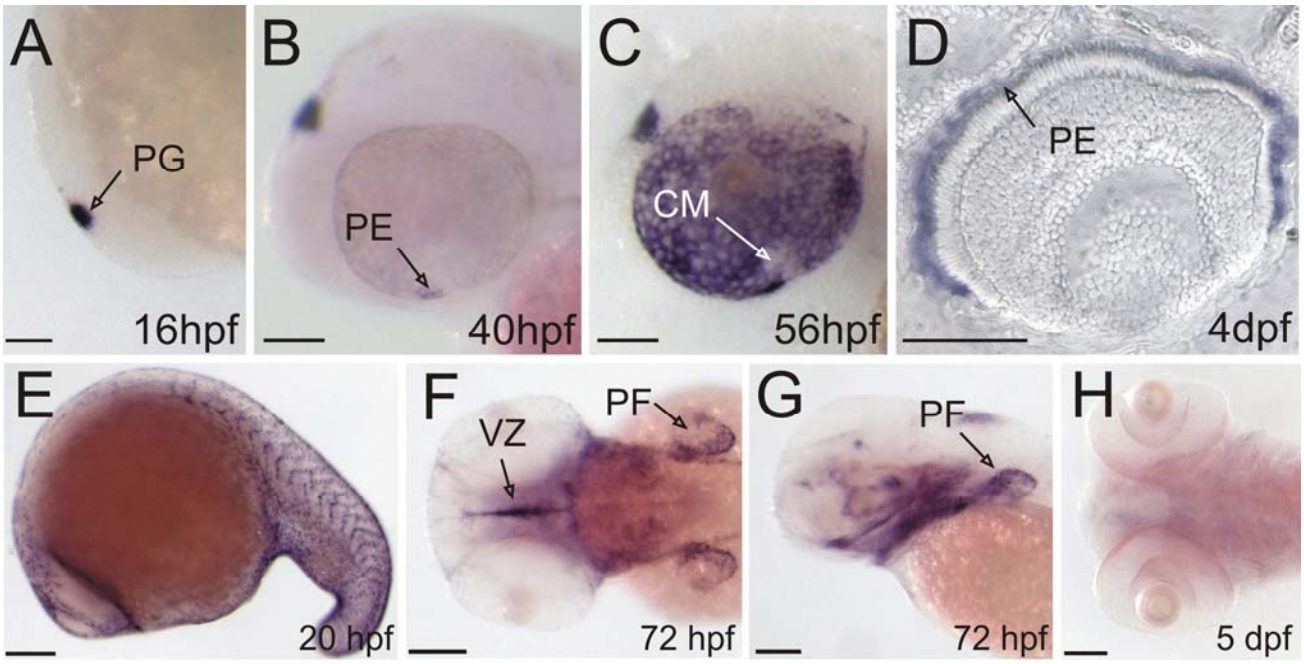

Fig. 1. Expression of *RPE65a* and *RPE65b* at different developmental stages. *A–C,E,G*, lateral views, anterior toward the left. *D*, transverse section through the eyes of 4 dpf larvae. *F,H,* dorsal views, anterior towards the left. *A–D,* Expression of *RPE65a*. *A*, Beginning at 16 hpf, *RPE65a* is expressed in the pineal gland. *B,C*, At 40 hpf, *RPE65a* expression starts in the ventral nasal patch of the eyes and spreads over the whole RPE at later developmental stages. *D*, Transverse section of a 4 dpf larval eye reveals that *RPE65a* expression is confined to the pigment epithelium. *E-H*, Expression of *RPE65b*. E, During segmentation *RPE65b* is expressed in migrating neural crest cells. F,G, *RPE65b* mRNA is detectable in the developing pectoral fin, the ventricular zone and the upper and lower jaw in early larval stages. *H*, *RPE65b* expression fades out in later stages. CM, ciliary margin; PE, pigment epithelium; PF, pectoral fin; PG, pineal gland; VZ, ventricular zone. Scale bars represent 100 μm.

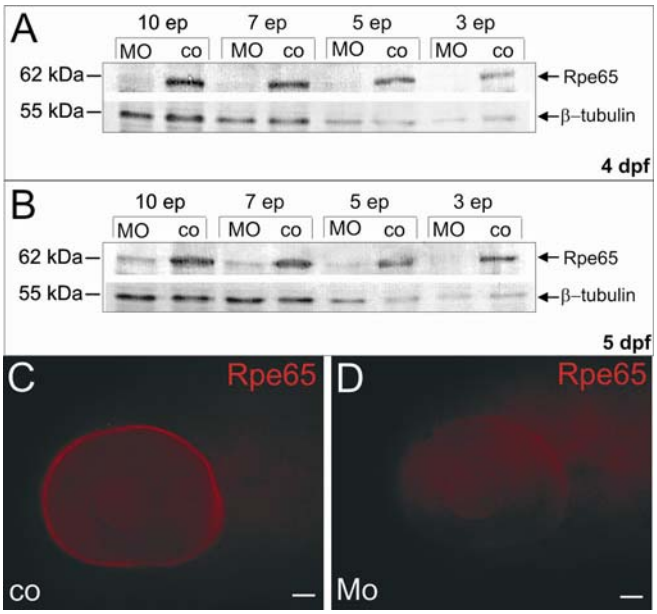

Fig. 2. MO-treatment induces RPE65-deficiency in 5 dpf larvae. *A,B*, Immunoblot blot analysis for RPE65 in protein extracts derived from 10, 7, 5 and 3 heads of 4- and 5-day-old *RPE65*-morphants and controls; beta-Tubulin was used as a loading control. *C,D*, Epi-fluorescence micrographs of whole-mount immunostainings with an antiserum raised against zebrafish RPE65 confirmed RPE65-deficiency in the eyes of 5 dpf MO-treated larvae. Scale bars represent 50 μm. ep, eye pair.

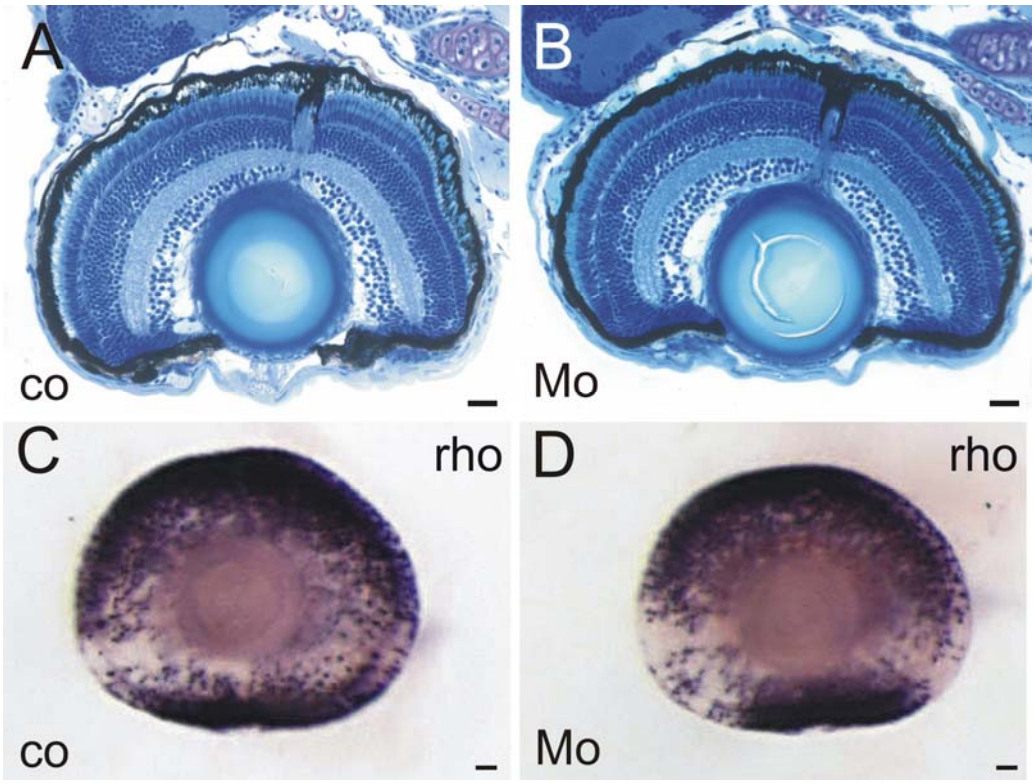

Fig. 3. Morphology of the eyes of *RPE65a* morphants. *A*,*B*, Transverse sections through the eyes of 6 dpf larvae revealed a normal stratification of the distinct retinal layers. *C*,*D*, Lateral views on the eyes (dorsal on the top, ventral at the bottom, anterior to the left) of 5 dpf larvae stained for *rhodopsin* mRNA expression. Rods show a comparable number and distribution in RPE65a morphant and control embryos. Scale bars are 20 µm. Mo, *RPE65a* morphant, co, control sibling.

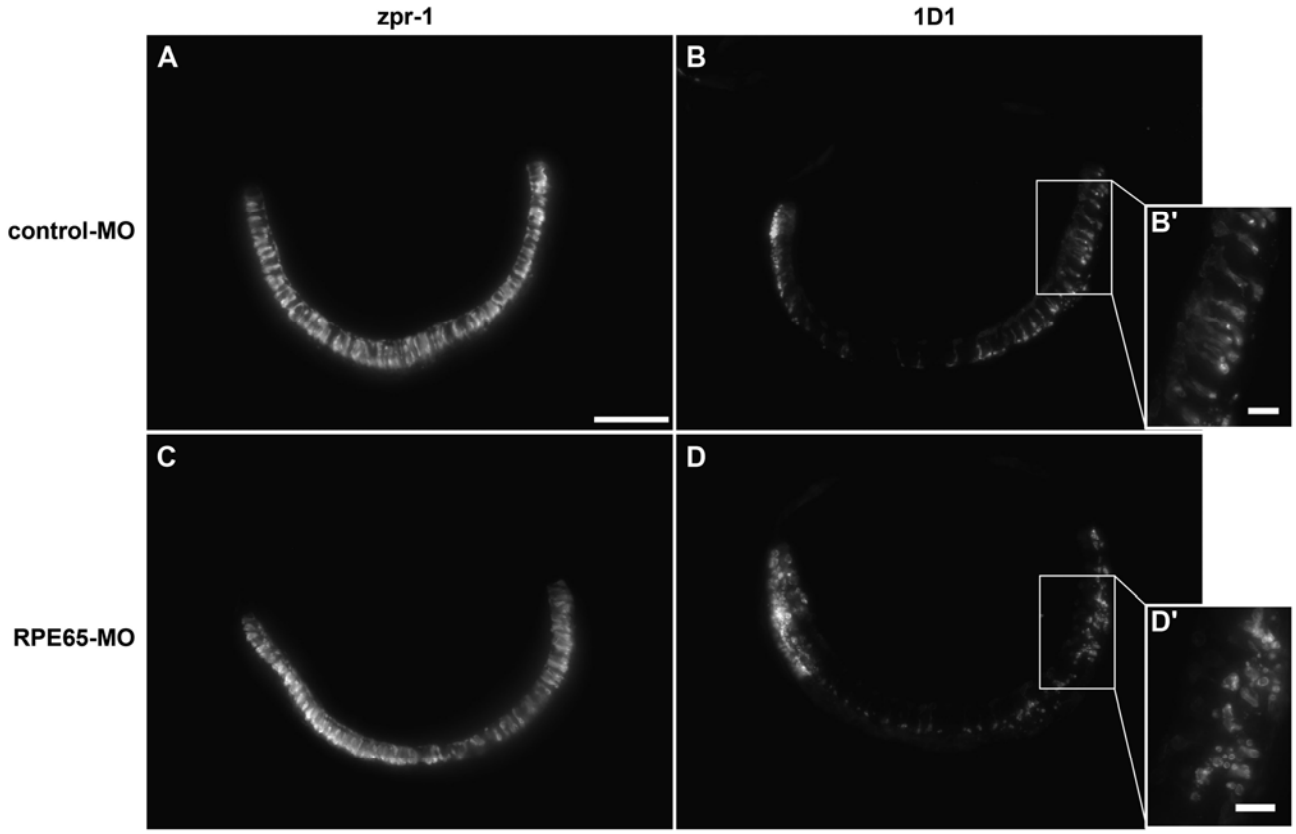

Fig. 4. Maximal intensity projections of immunostainings for zpr1 (double cones) and 1D1 (rods) in transverse sections of 5dpf control and *RPE65a* morphant larvae. *A,C,* Zpr1-positive double cones are strongly labeled throughout the entire retina of morphant and control larvae. *B,D,* 1D1-positive rods show the same distribution pattern in both, control and morphant retinas. *B',* Higher magnification of the dorsal part of control larva shows normal rod photoreceptor morphology. *D',* RPE65a-deficient larva shows deteriorating rod outer segments breaking up into small particles, presumptive phagosomes. co, control larva; Mo, *RPE65a* morphant larva. Scale bars: A 50 μm, B'+D' 10μm.

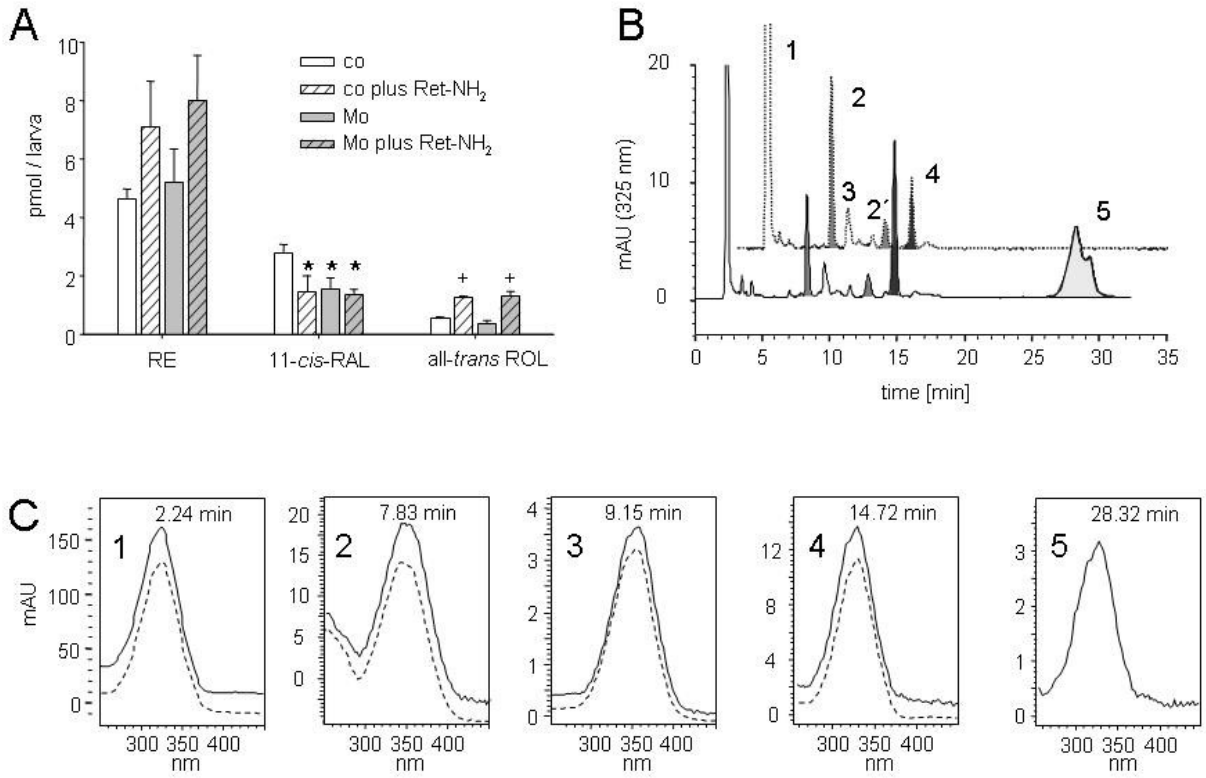

Fig. 5. Levels of retinoids in 5 dpf larvae subjected to Mo and/or Ret-NH_2 treatment. *A*, Retinoid composition of over night dark adapted - illuminated (10,000 lux for 20 min) and dark-readapted (45 min) 5 dpf larvae subjected to Mo and/or Ret-NH_2 treatments. The values give the average of 3 independent experiments for each condition with 75 larvae. * Significant difference to untreated control larvae (Student's *t* test, $P<0.02$); + significant difference to the corresponding control (Student's *t* test, $P<0.01$) *B*, HPLC-chromatograms of lipophilic extracts of heads of 5 dpf larvae subjected to Ret-NH_2 treatment (lower trace) as compared to untreated controls (upper trace). *C*, Spectral characteristics of retinoids (solid) from zebrafish larvae as compared to authentic standards (dashed). 1, RE; 2, 11-*cis*-RAL oxime (*syn*), 2', 11-*cis*-RAL oxime (*anti*), 3, all-*trans* RAL (*syn*); 4, all-*trans* ROL: 5, retinylamide. co, control larva; Mo, *RPE65a* morphant larva.

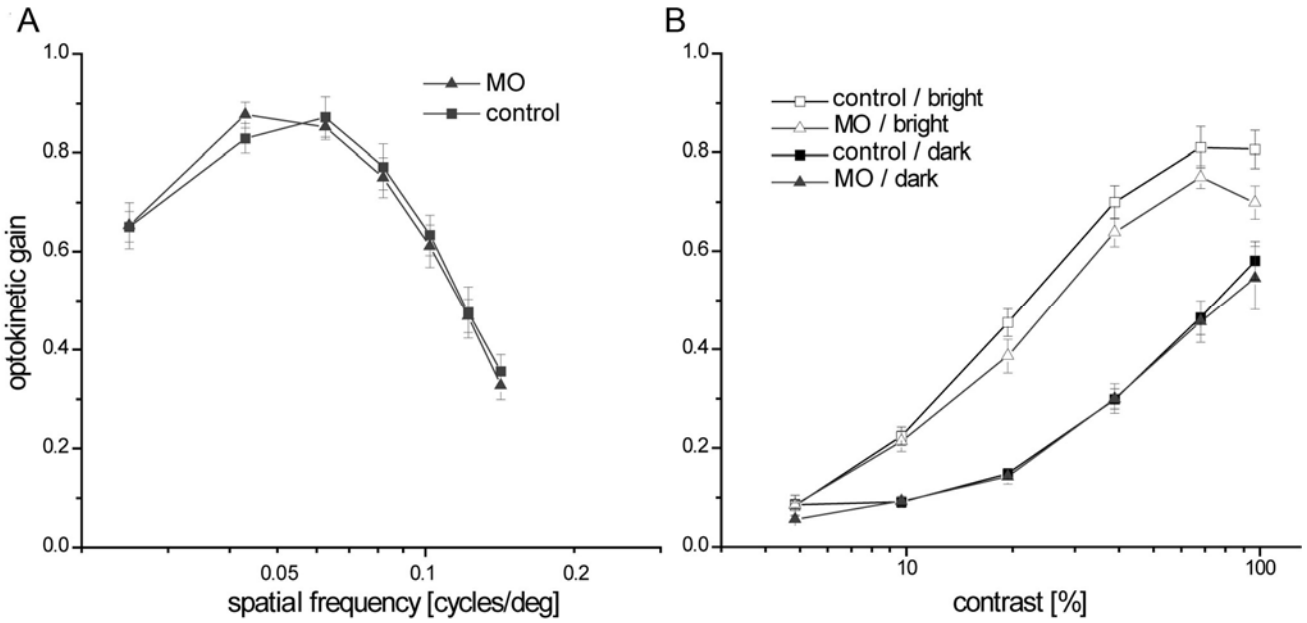

Fig. 6. Behavioral analysis of RPE65-deficient larvae. *A*, Optokinetic gain triggered by moving gratings velocity = 7.5 deg/s, contrast = 99%) of varying spatial frequency under moderate background intensity 120 cd/m2) shows no significant reduction in visual acuity in RPE65 morphants (n=8) compared to controls (n=8). *B*, Optokinetic gain measured with moving gratings (velocity = 7.5 deg/s, spatial frequency = 0.06 cycles/deg) of varying contrast. Contrast sensitivity of the optokinetic response is not significantly reduced in RPE65-deficient larvae when measured with a dark grating stimulus (n=5, 0.36 cd/ m2, closed symbols). Under high intensity conditions (n=5, 386 cd/ m2, open symbols) optokinetic gain is slightly reduced (2-way ANOVA with repeated measures on factor contrast $p < 0.05$).